基础研究

适用于犬输尿管脱细胞基质制备的灌注系统

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摘要:目的 探讨应用灌注系统制备输尿管脱细胞基质的可行性方案。**方法** 利用输尿管本身存在的管腔结构,通过灌注系统制备输尿管脱细胞基质。根据化学洗涤剂的不同特性设置3个不同的灌注组,单纯SDS组、单纯TritonX-100组以及联合组(TritonX-100+SDS)。利用HE染色,DAPI染色,DNA定量,琼脂糖凝胶电泳评价输尿管脱细胞基质中细胞核的残留情况。采用Masson's 3色染色、Alcian Blue染色、胶原含量测定、GAG含量测定、扫描电镜和毒性检测等手段评价制备输尿管脱细胞基质的三维结构和生物活性成分。**结果** HE染色和DAPI染色显示联合组制备的输尿管脱细胞基质中没有明显的核物质残留,DNA定量和凝胶电泳证实了这一结果。Masson's 3色染色、Alcian Blue染色、胶原含量测定和GAG含量测定显示联合组保留了输尿管脱细胞基质三维胶原结构和生物活性成分。扫描电镜观察联合组制备的输尿管脱细胞基质表面存在大量的孔隙结构。毒性测定证实联合组制备的输尿管脱细胞基质无毒性。**结论** 本研究灌注系统可用于输尿管脱细胞基质的制备,筛选出一套比较理想的脱细胞方案,制备的输尿管脱细胞基质可用于输尿管组织工程再造。

关键词:输尿管;脱细胞基质;灌注系统;组织工程;支架

A perfusion system for preparing canine ureteral acellular matrix

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Abstract: Objective To explore the feasibility of preparing ureteral acellular matrix (UAM) using perfusion systems. **Methods** Using the luminal structure of the ureter, the UAM was prepared by perfusing canine ureter with SDS, TritonX-100, or both. The residual nuclei in the UAM were evaluated using HE staining, DAPI staining, DNA quantification, and agarose gel electrophoresis. The three-dimensional ultrastructure and the bioactive components were evaluated by Masson's trichrome staining, Alcian Blue staining, collagen quantification, GAG quantification, scanning electron microscopy (SEM), and toxicity detection. **Results** HE staining and DAPI staining showed the absence of obvious nuclear materials in the combined group, which was further confirmed by DNA quantification and agarose gel electrophoresis. Masson's trichrome staining, Alcian Blue staining, collagen quantification and GAG quantification all verified that the ultrastructure and the bioactive components were well preserved in the combined group. SEM showed a large amount of porous structure on the surface of the UAM prepared by combined perfusion, and toxicity assay confirmed that the prepared UAM was nontoxic. **Conclusion** Perfusion of canine ureter with SDS and TritonX-100 is feasible to prepare UAM for ureteral reconstruction.

Key words: ureter; perfusion system; acellular matrix; tissue engineering; scaffold

泌尿系统的肿瘤、炎症、损伤和先天畸形等疾病都可导致输尿管损伤[1],传统的手术治疗方式不一定能完全恢复输尿管自身的功能,甚至可能会引起一系列的手术并发症,如再发性狭窄、输尿管漏、肾损伤等[2]。随着组织工程技术在泌尿系统中广泛应用,为输尿管的重建提供了新的契机[3]。在输尿管重建过程中,支架的选择尤为重要。支架材料主要包括人工合成支架材料和天然支架材料两种。人工合成支架材料缺乏良好的生物相容性,且不具备输尿管的蠕动功能,以及移植后形成硬壳,人工合成支架材料的应用受限[4]。天然支架材料中的脱细胞基质因其独特的优势,引起广泛的关注。本实验采用灌注系统结合脱细胞常规应用的物理和化学法,制备输尿管脱细胞基质,评价制备的输尿管脱细胞基质,探讨其应用于输尿管再造的可能性。

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1 材料和方法

1.1 实验动物和主要试剂

健康雌性10月龄比格犬5只,体质量7~10 kg,解放军总医院动物中心提供。高糖培养基(H-DMEM)、胎

牛血清(Gibco);Ⅳ型胶原酶、Dispase酶、十二烷基硫酸钠(SDS)、TritonX-100、4,6-二脒基-2-苯基吲哚(DAPI)(Sigma);DNA提取试剂盒(天根);犬糖胺聚糖Elisa试剂盒(上海邦奕生物科技有限公司);α-SMA单克隆抗体(北京博奥森公司)。

1.2 输尿管取材

比格犬静脉给予3%戊巴比妥钠(25 mg/kg)麻醉,气管插管。下腹正中切口进入腹腔,游离双侧全长的输尿管去除其周围粘连的组织后剪断取出,放在4 ℃的磷酸盐缓冲液(PBS)中。切开膀胱顶部,取2.5 cm×2.5 cm的全层膀胱组织。

1.3 灌注法制备输尿管脱细胞基质

将取材的输尿管放在−80 ℃冰箱中24 h后常温解冻,将16-G的针头插入到输尿管管腔中,通过硅胶管将输尿管连接到蠕动泵上,进行灌注脱细胞(图1A,B)。分别使用3组不同灌注方案进行灌注脱细胞处理。1% SDS 灌注 8 h(SDS 组);1% TritonX-100 灌注 8 h(TritonX-100组);1%TritonX-100灌注7 h后再用1% SDS灌注1 h(联合组)。去离子水灌注24 h洗去残留的灌注液。灌注速度保持在1.5 mL/min。制备好的输尿管脱细胞基质浸泡在包含有100 U/mL青霉素和100 μg/mL的链霉素的PBS中,4℃储存。

1.4 输尿管脱细胞基质组织学观察

HE染色观察输尿管脱细胞基质中是否有细胞核的残留,DAPI染色进一步观察细胞核残留情况,Masson's 3色染色观察胶原的分布和走向,Alcian Blue染色观察输尿管脱细胞基质中糖胺聚糖(GAG)的分布情况[5]。

1.5 输尿管脱细胞基质DNA定量分析

将输尿管和输尿管脱细胞基质分别冻干称重后按照DNA试剂盒说明书中步骤离心纯化DNA。用分光光度计来测量DNA的含量。同时配置2%的琼脂糖凝胶,进行凝胶电泳观察DNA条带。

1.6 输尿管脱细胞基质生物活性成分定量检测

将输尿管和输尿管脱细胞基质分别冻干称重,利用酸溶解法测量胶原含量[6]。利用GAG试剂盒测量GAG含量,按照说明书上步骤来提取GAG,酶标仪测量525 nm处的吸光度[7],计算GAG含量。

1.7 输尿管脱细胞基质扫描电镜观察

分别用扫描电镜观察输尿管脱细胞基质的管腔面和管壁面。将输尿管脱细胞基质从管腔中间剪开,展开成片状。用2.5%戊二醛室温固定后入梯度酒精脱水,CO_2干燥箱干燥样本,喷金后扫描电镜观察[8]。

1.8 膀胱平滑肌细胞分离鉴定

膀胱组织去除黏膜和黏膜下层,将平滑肌层剪碎并用Ⅳ型胶原酶和Dispase酶在37 ℃消化30 min,离心,重悬接种到培养皿上培养[9]。取第3代的平滑肌细胞做细胞爬片,平滑肌特异性蛋白α-SMA作为一抗,进行荧光染色并观察。

1.9 输尿管脱细胞基质的毒性检测

将制备的输尿管脱细胞基质浸泡在H-DMEM培养基中72 h制备浸提液[10]。第3代犬膀胱平滑肌细胞接种在24孔板上,种植密度为每孔2×10^4细胞。用H-DMEM培养基培养24 h后改换浸提液作为培养基进行培养,同时将细胞培养在H-DMEM上作为对照组。分别在1,3,7 d用AO/PI染色观察细胞凋亡情况。在1,3,5,7 d进行MTT实验,观察细胞的增殖情况。

1.10 统计学处理

所有数据结果均用SPSS 17.0进行分析处理,数据以均数±标准差表示。胶原和GAG含量各组之间的差异情况采用Mann-whitney U检验统计分析。检验水准:α=0.05,$P<0.05$认为差异有统计学意义。

2 结果

2.1 输尿管脱细胞基质的大体形态观察

脱细胞过程中输尿管组织从最初的红色逐渐变为白色(图1C,D)。

2.2 输尿管脱细胞基质组织学检测

HE染色显示SDS组和TritonX-100组制备的输尿管脱细胞基质有核样物质的残留,联合组中没有观察到核样物质的残留(图2A~D)。DAPI染色进一步证实了HE染色结果(图2E~H)。Masson's 3色染色显示3组胶原结构均保存良好(图3A~D)。Alcian Bluer染色显示3组中均保留有GAG(图3E~H)。





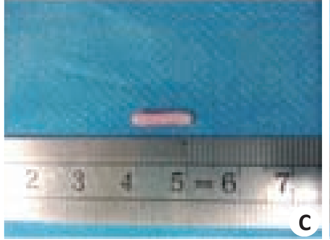




图1 灌注系统组成及输尿管脱细胞前后大体形态

Fig.1 The perfusion system (*A*, *B*) and the canine ureter before (*C*) and after (*D*) decellularization (*C*-*D*).

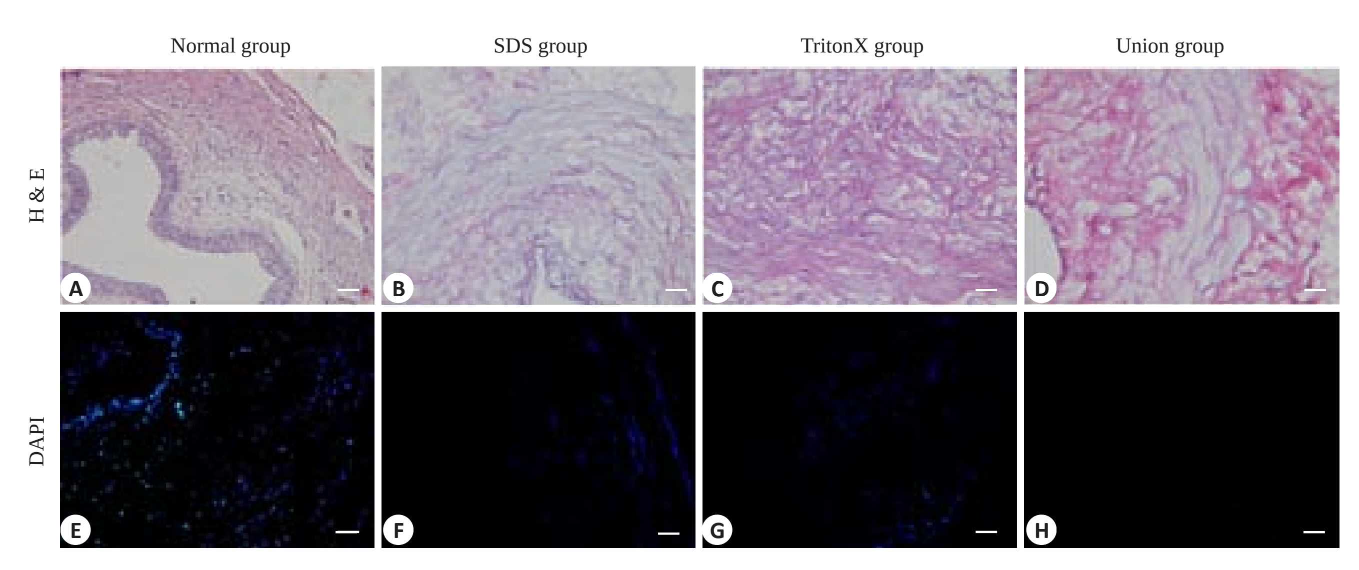


图2 输尿管以及3组不同灌注方案制备输尿管脱细胞基质的HE和DAPI染色

Fig.2 HE staining (*A-D*) and DAPI staining (*E-H*) of the ureter and the UAM prepared by three different perfusion protocols. Scale bar=100 μm.

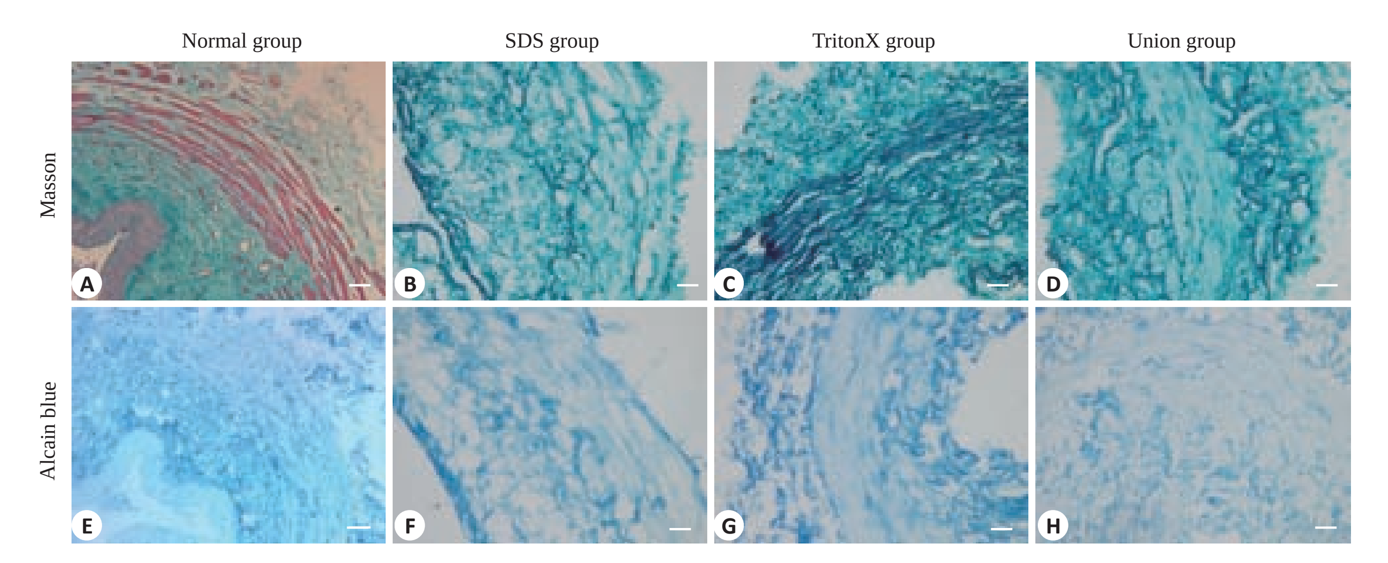


图3 输尿管以及3组不同灌注方案制备输尿管脱细胞基质Masson's 3色和Alcian Blue染色

Fig.3 Masson's trichrome staining (*A-D*) and Alcian Blue staining (*E-H*) of the ureter and the UAM prepared by three different perfusion protocos. Scale bar=100 μm.

2.3 输尿管脱细胞基质中DNA含量分析

对比正常组输尿管(1760.8±190.1 ng/mg),联合组(40.3±3.5 ng/mg)中核样物质基本被清除,而另外两组中则残留较多的核样物质(SDS组352.5±30.7 ng/mg;Triton-100组951.7±63.4 ng/mg)(图4B)。2%琼脂糖凝胶电泳显示TritonX-10组可以看到明显的条带,SDS组中可看到条带但不明显,联合组中则完全看不到条带(图4A)。

2.4 输尿管脱细胞基质中胶原和GAG含量检测

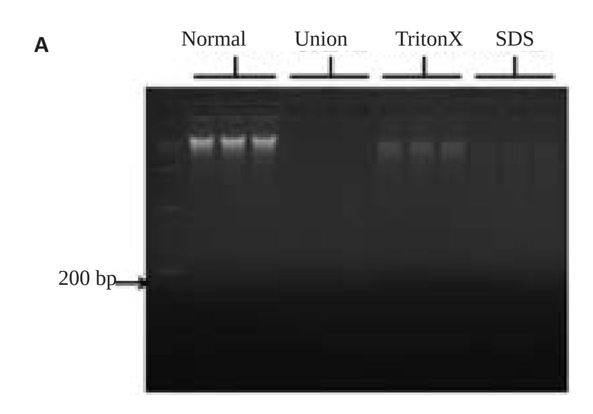
胶原含量测定显示SDS组(212.73±12.97 μg/mg)胶原含量较正常组输尿管(284.47±19.60 μg/mg)含量降低,且有统计学差异。联合组(265.89±17.74 μg/mg)和TritonX-100组(270.86±21.36 μg/mg)胶原含量较正常组输尿管无明显差别(图5A)。GAG定量检测显示3组GAG含量较正常组输尿管(3.613±0.165 μg/mg)均降低且有统计学差异,TritonX-100组(1.780±0.150 μg/mg)和联合组(1.726±0.148 μg/mg)之间无统计学差异,SDS组(1.001±0.091 μg/mg)GAG含量相比联合组明显下降,且有统计学差异(图5B)。

2.5 输尿管脱细胞基质的扫描电镜观察

联合组制备的输尿管脱细胞基质电镜照片显示管腔面和管壁面的胶原纤维构成三维网络结构且包含有大量的孔隙。而输尿管本身的管腔面和管壁面结构致密无孔隙结构存在(图6A~D)。

2.6 膀胱平滑肌细胞鉴定

α-SMA蛋白为平滑肌细胞特异性蛋白。免疫荧光染色显示分离的细胞特异性的表达α-SMA蛋白(图7A,B),结合分离的部位确定所分离的细胞为平滑肌细胞。



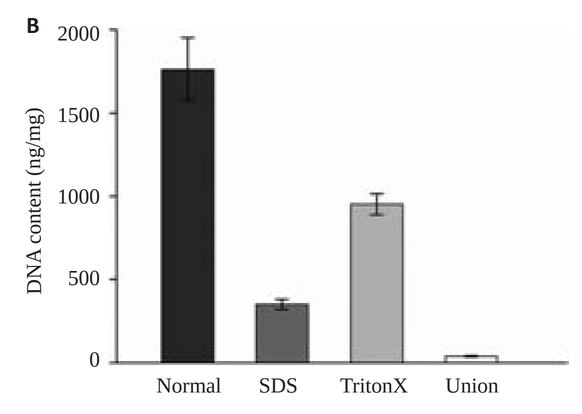
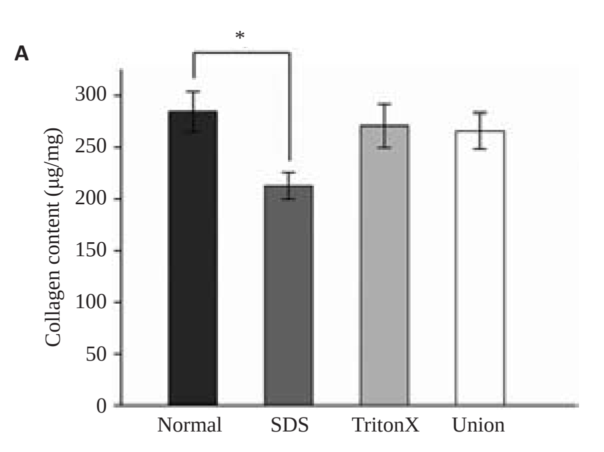


图4 DNA琼脂糖凝胶电泳以及DNA定量

Fig.4 Agarose gel electrophoresis and DNA quantification. DNA agarose gel electrophoresis showed no visible DNA bands in the combined group (*A*). DNA quantification also confirmed that the cellular material was effectively removed in the combined group (*B*).



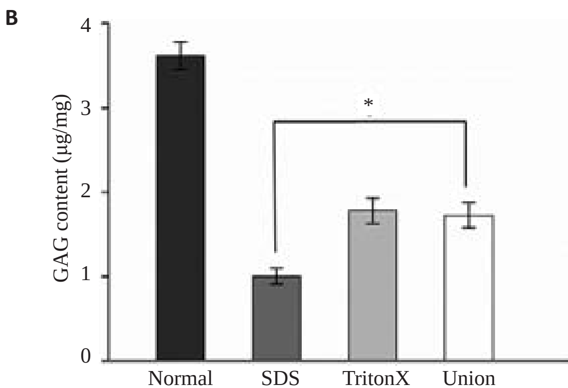


图5 胶原和GAG定量分析

Fig.5 Collagen and GAG quantitative analysis. Collagen quantification showed no significant difference between the normal group and the combined group (*A*). GAG assay showed a lower GAG content in SDS group than in the combined group (B). *$P<0.05$.

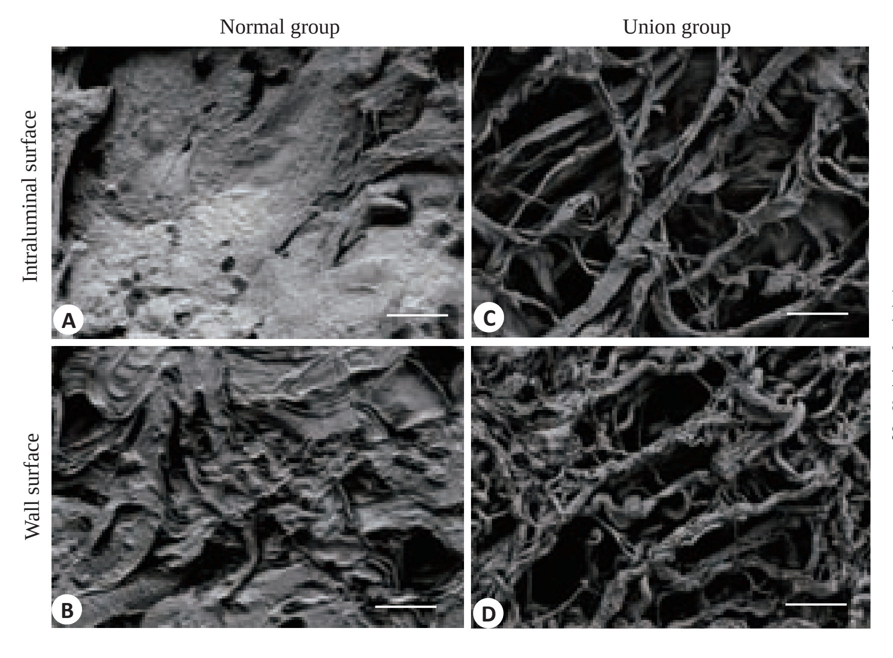


图6 输尿管和联合组制备输尿管脱细胞基质的扫面电镜观察

Fig.6 SEM images of the ureter and UAM prepared by combined perfusion. *A*, *C*: Intraluminal surface; *B*, *D*: Tube wall surface. Scale bar=20 μm.

2.7 输尿管脱细胞基质毒性检测

AO/PI染色结果显示细胞在浸提液组中生长良好，未出现明显的凋亡。MTT实验证实细胞在输尿管浸提液中仍保留增殖能力(图8)。结果表明输尿管脱细胞基质无化学洗涤剂残留，无毒性。

3 讨论

近年来，随着内镜技术在临床中的广泛应用，输尿管损伤的发生机率逐年升高。传统的手术治疗效果往往不甚理想。组织工程技术为输尿管的修复重建带来了新的思路。随着组织工程技术的发展，天然组织的细

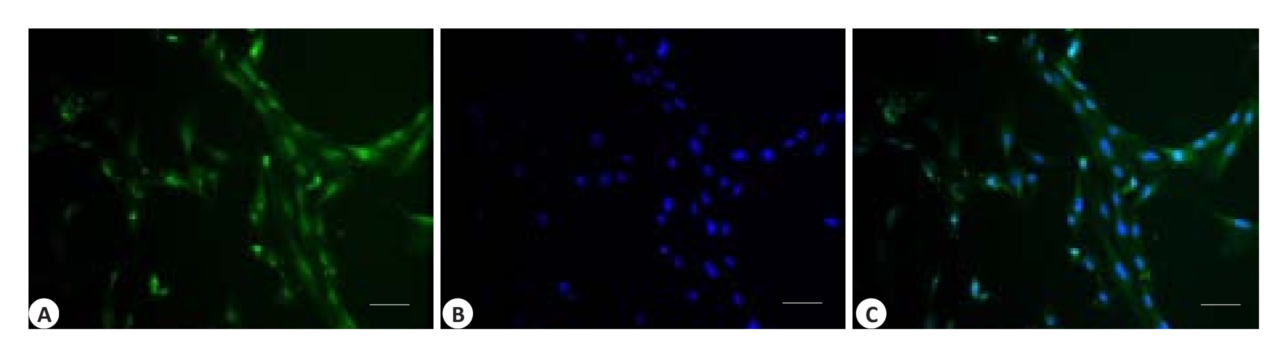


图7 平滑肌细胞荧光染色

Fig.7 Fluorescent staining of smooth muscle cells. The smooth muscle protein was stained green (*A*) and the nuclei were stained blue (*B*). Scale bar=100 μm; *C*: Merge.

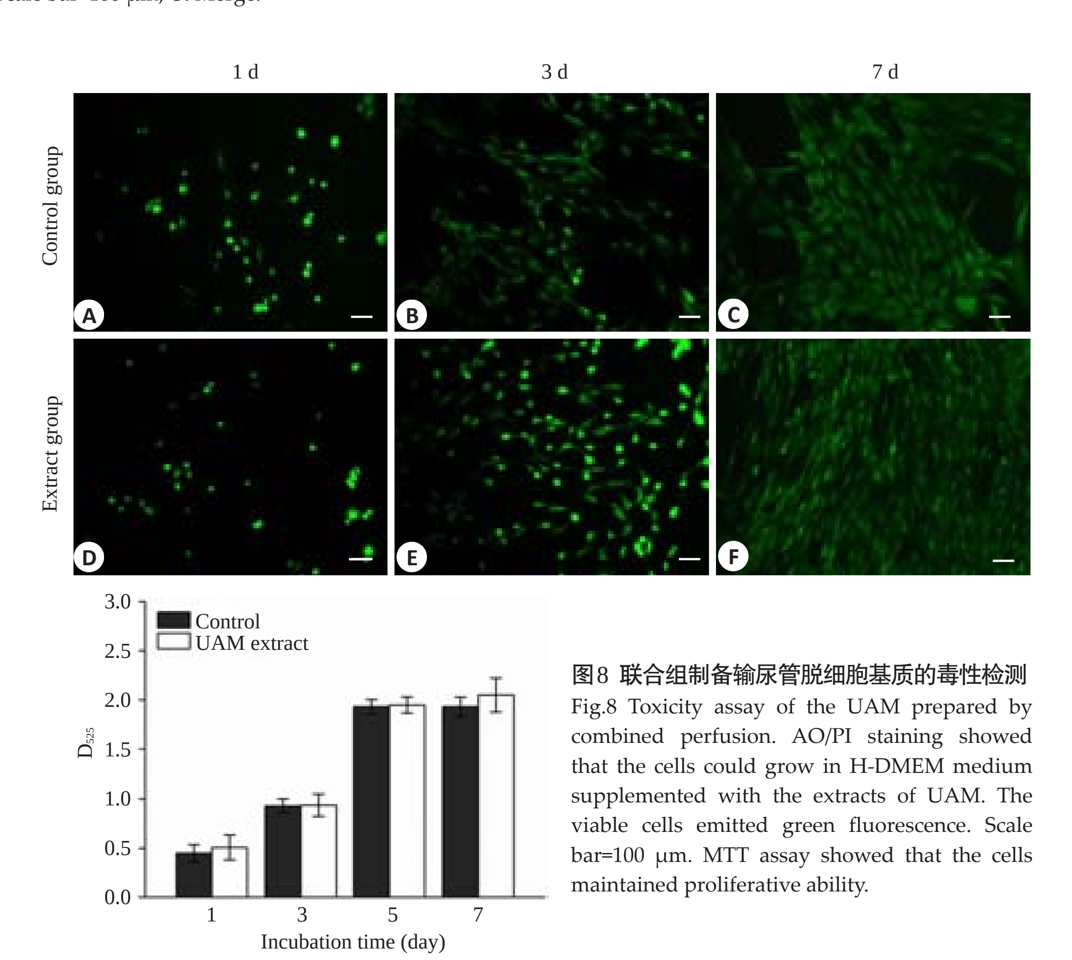


图8 联合组制备输尿管脱细胞基质的毒性检测

Fig.8 Toxicity assay of the UAM prepared by combined perfusion. AO/PI staining showed that the cells could grow in H-DMEM medium supplemented with the extracts of UAM. The viable cells emitted green fluorescence. Scale bar=100 μm. MTT assay showed that the cells maintained proliferative ability.

胞外基质(ECM)因起所具有的独特优势受到了越来越广泛的关注。ECM的成分和组成非常独特,在不同的组织器官中细胞外基质的构成也不尽相同,但其共同特点是利于组织器官特异性细胞的生长,且能维持组织或器官的正常功能[11]。

输尿管细胞外基质的常规制备方法[4, 12-13]是利用持续搅拌的物理方法结合化学洗涤剂进行,其缺点是制备过程需要花费几天甚至一周的时间,且长时间的搅拌和洗涤剂持续作用会对输尿管组织结构造成严重破坏,并会导致细胞外基质的生物活性成分的丢失。本研究采用灌注系统来制备输尿管脱细胞基质,使输尿管脱细胞时间大为缩短,且避免了长时间的物理搅拌对输尿管结构和生物活性成分的破坏。灌注系统是利用组织或器官本身的脉管或管腔结构来推动脱细胞液流过整个的组织或器官,在这一过程中带走细胞核成分,同时维持结构的完整性。灌注系统已广泛应用于组织器官脱细胞基质的制备,例如心脏[14]、肺脏[15]、肝脏[16]及肾脏[17],且取得了很好的脱细胞效果。本研究利用输尿管本身存在的管腔结构,将输尿管连接到灌注系统进行脱细胞基质的制备,脱细胞过程完全符合输尿管本身的生理功能。

Crapo等[18]曾指出任何一种细胞成分清除剂和方法都会改变细胞外基质的成分并会造成不同程度的微观结构破坏,我们应当最小程度的降低这些破坏而不是避免。研究过程中我们先将输尿管快速冷冻,这一过程会使细胞内形成冰晶损坏细胞膜使细胞碎裂,但不影响

组织结构,同时利于洗涤剂的渗透[19]。Gilbert等[20]研究报道了非离子型洗涤剂TritonX-100作用温和对组织结构损伤小,且对较厚的组织有较强的细胞清除能力。而离子型洗涤剂SDS对细胞成分的清除能力很强,但会对组织结构造成严重破坏并能清除生长因子[18]。实验过程中我们结合两种洗涤剂的不同特性,首先使用作用温和的TritonX-100持续作用于输尿管组织,这一过程中厚而致密的输尿管组织逐渐变得疏松,更加有利于洗涤剂的渗透。后换用脱细胞能力强的SDS,短时间作用于输尿管组织达到完全的脱细胞效果,同时最小程度地破坏输尿管组织的微观结构和生物活性成分。

用于输尿管修复重建的脱细胞基质应该具备两个基本特征[21]:(1)引起免疫反应的细胞核成分被清除,(2)本身的三维结构和生物活性成分得到很好地保留。我们对制备的输尿管脱细胞基质进行了详细的评价。HE染色和DAPI染色结果证实联合组制备的输尿管脱细胞基质细胞核成分几乎被完全移除,且清除效率明显优于另外两组,DNA定量检测和琼脂糖凝胶电泳进一步佐证了这一结果。在三维结构和生物活性成分的保留上,胶原含量测定显示联合组制备的输尿管脱细胞基质与正常组输尿管中胶原含量无差别,而SDS组胶原含量明显降低,表明长时间的SDS作用会导致胶原结构的损伤。GAG定量检测显示联合组制备的输尿管脱细胞基质GAG含量仅为正常输尿管的一半,其减少程度在统计学上有差异,考虑是因为GAG对洗涤剂的高度敏感,导致GAG在脱细胞过程中损失[22]。扫描电镜证实联合组制备的输尿管脱细胞基质表面存在大量的孔隙结构,利于细胞的植入和渗透,而毒性实验也表明制备的输尿管脱细胞基质没有毒性。这些结果证实联合组制备的输尿管脱细胞基质可以用于输尿管修复重建实验研究。

综上所述,本研究证实灌注系统适用于输尿管脱细胞基质的制备,且耗时较短。筛选出一套较为理想的输尿管脱细胞方案,制备的输尿管脱细胞基质细胞核成分几乎被完全清除,三维结构和生物活性成分很好地被保留。输尿管脱细胞基质可以进一步用于输尿管修复重建研究。

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